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Bioleaching of pyrite by iron - oxidizing acidophiles under the influence of reactive oxygen species

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Abstract

After 24h of exposure to acidic media, pyrite generates reactive oxygen species (ROS). Freshly-crushed pyrite with grain sizes between 50-100 µm at 5 % (w/v) pulp density generated 0.17 ± 0.01 mM H_2O_2, while 10% pyrite generated 0.29 ± 0.01 mM and 30 % pyrite generated 0.83 ± 0.06 mM. These levels of H_2O_2 probably inhibited iron oxidation in iron-grown cells of *Acidithiobacillus ferrooxidans*^{T} but not in pyrite-grown cells. ROS originating from pyrite, which was incubated for 24 h in acidic medium, likely prohibited pyrite dissolution by iron-grown cells, while pyrite-grown cells were probably adapted to these concentrations of ROS. Periodical addition of 100 µM H_2O_2 to pyrite cultures inoculated with pyrite-grown cells did not decrease iron dissolution. By high throughput proteomics analysis, an increased expression of proteins related to oxidative stress management, iron- and sulfur oxidation systems, carbon fixation and biofilm formation was observed in biofilm cells grown on pyrite compared to iron-grown cells.

Introduction

In acidic conditions, sulfide minerals, especially pyrite, generate toxic ROS, resulting in oxidative stress of acidophilic metal oxidizing microorganisms [1, 2]. Pyrite spontaneously generated H_2O_2, $(O_2^{\bullet})^-$and $OH^{\bullet}$ radicals in aqueous solution either in the presence or in the absence of oxygen [3, 4]. As a result, bioleaching bacteria are likely to be confronted with high levels of oxidative stress. It is indicated that in comparison with iron-grown cells, pyrite- grown cells of *A. ferrooxidans*^{T} are better adapted to H_2O_2 [5]. Studies on the oxidative stress response of acidophiles have been carried

out [6, 7], though the influence of ROS on bioleaching and strategies to withstand ROS of *A. ferrooxidans*^{T} are still inadequately understood. The aim of this study was to characterize the effect of H_2O on *A. ferrooxidans*^{T} grown with iron or pyrite as growth substrate and subsequently on pyrite bioleaching efficiency. High throughput proteomics analysis was used to get insights into the mechanisms for ROS tolerance in *A. ferrooxidans*^{T}.

Materials and Methods

Bacteria and cultivation conditions. *A. ferrooxidans*^{T} was grown in Mackintosh medium [8] adjusted to pH 1.8, supplied either with ferrous iron 3 gL^{-1} or pyrite at 5% w/v (50-100 µm grain size); cultures were incubated at 28^0C and shaking at 120 rpm.

Pyrite grains. Pyrite was prepared as described [9] and sterilized by autoclaving at 120^0C for 4h under N_2 atmosphere.

Quantification of H_2O_2: H_2O_2 was quantified using the spectrophotometric determination method of Baga [10].

Iron determination. Pyrite dissolution was assessed by quantification of ferrous and total iron concentration using the 1,10-phenathroline method according to German standards [5].

Proteomics analysis. Proteins from biofilm cells grown on pyrite surfaces and cells grown on ferrous iron after 5 days of incubation were extracted, purified, and analyzed as described [11].

Results and Discussion

Generation of H_2O_2 in pyrite-containing acidic MAC media. Table 1 summarizes the concentrations of H_2O_2 produced by pyrite after 24 h incubation in MAC media. Obviously, pyrite generated H_2O_2 in dependence of pyrite grain size and pulp density. Pyrite with the grain size of 50-100 µm generated higher concentrations of H_2O_2 than the fraction with 100-200 µm grain size. Also, pulp densities of 30% (w/v) generated H_2O_2 concentrations 5 times higher than that of 5% (w/v).

Tab. 1. H_2O_2 generation (mM) by pyrite of different grain size and pulp density after 24h in MAC medium.

	Pulp density % (w/v)		
Grain sizes (µm)	5	10	30
50-100	0.17 ± 0.01	0.29 ± 0.01	0.83 ± 0.06
100-200	0.05 ± 0.00	0.09 ± 0.01	0.25 ± 0.01

About 0.9 mM H_2O_2 is formed by a pyrite load of 10% (pyrite sizes <106 µm) at pH 4.5 and more H_2O_2 is formed at lower pH [12]. The H_2O_2 generated in this study was lower than in the study by Nooshabadi et al [12] . However, H_2O_2 concentrations of ≥ 50µM have significant toxic effects on *A.ferrooxidans* [13, 14]. Therefore, 5% (w/v) pyrite loading, a size of 50-100, generates toxic levels of H_2O_2 for *A.ferrooxidans*.

Pyrite dissolution. Figure 1 shows the dissolution of pyrite by *A.ferrooxidans*^{T}. Total concentration of iron in solution without the preincubation of pyrite was nearly two times the total iron concentration in solution with pyrite-generated H_2O_2 in the case of iron-grown cells (**a**). In contrast, there was no significant reduction in pyrite dissolution when pyrite-grown cells were used (**b**).

Also, in case of pyrite-grown cells no significant differences in pyrite dissolution assays with the periodical additions of external H_2O_2 concentrations occurred (Figure 2). In abiotic assays, with and without addition of H_2O_2, the total iron ions remained relatively constant at low concentration.

In biotic assays, regardless of the addition of external H_2O_2, total iron concentrations increased steadily and reached approximately 45 mM after 28 days of incubation.

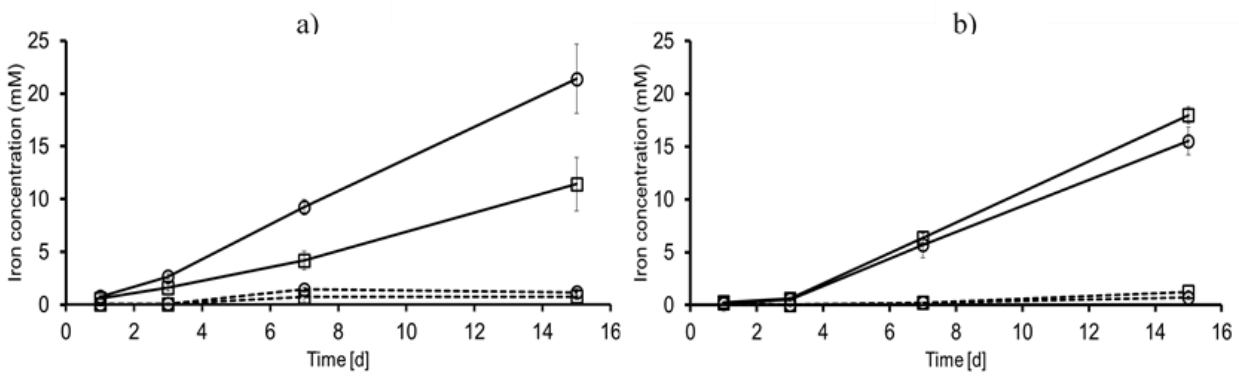

Fig. 1. Pyrite dissolution with a) iron-grown cells and b) pyrite-grown cells of *A.ferrooxidans*^{T}. Control without preincubation of pyrite (*circles*) and assay with 24h preincubation of pyrite in MAC media (*squares*) were compared. Iron ion concentrations include ferrous iron (*dashed line*) and total iron (*solid line*). The initial cell inoculation was $5*10^7$ cells/mL. All assays in experiment were performed in triplicate; error bars show standard deviation of the mean as indicated.

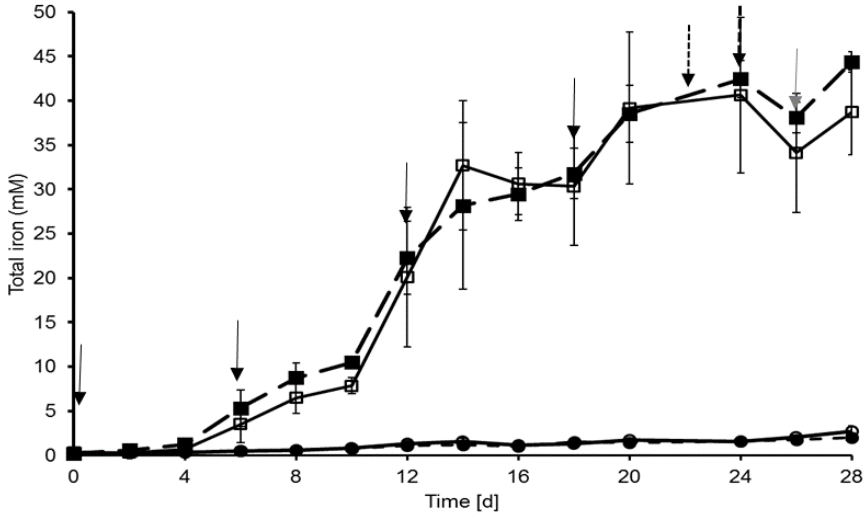

Fig. 2. Effect of external addition of H_2O_2 on pyrite dissolution using pyrite grown-cells of *A.ferrooxidans*^{T}. The additions of H_2O_2 were 100 µM at day 0, 6, 12 and 18 (*black arrows*), 500 µM at day 22, 24 (*dotted black arrows*) and 1 mM at day 26 (*grey arrows*). Total iron concentrations were measured in sterile (*circles*) and inoculated (*squares*) pyrite dissolution assays, with (*empty symbols*) and without (*filled symbols*) periodic addition. The initial cell inoculum was $5*10^7$ cells/mL. All assays in experiment were performed in triplicate; error bars show standard deviation of the mean as indicated.

Those results are similar to previous findings, indicating that compared to iron-grown cells, pyrite -grown cells of *A.ferrooxidans*^{T} were probably adapted to the presence of elevated levels of ROS, which are generated on metal sulfide surfaces [5]. Additionally, although H_2O_2 is commonly known as a toxic agent, at low levels it may also function as signaling agent [15]. Also, pre-exposure to low concentrations of H_2O_2 allows microorganisms to resist efficiently detrimental effects of oxidative stress [16]. Genetic response is of importance among strategies to protect enzymes and DNA against oxidative stress damage [16]. Bioleaching acidophiles possess several proteins responsible for coping with oxidative stress, involving several oxygen detoxification and repair

systems [6]. Proteomic data showed that during the growth with pyrite as energy source, cells of *A.ferrooxidans*^{T} utilized ROS degradation, redox balance, macromolecule repair mechanisms, metal and oxygen homeostasis as fundamental adaptation strategies to the elevated presence of ROS (data not shown). Also, our shot- gun proteomics analysis indicated that there was a remarkable increase in the expression of proteins related to oxidative stress response in biofilm cells grown on pyrite surfaces compared to iron-grown cells of *A.ferrooxidans*^{T}.

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